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In order to determine the feasibility of using gene therapy for breast cancer prevention in the rat, lacZ recombinant adenoviral and retroviral vectors were infused into the mammary glands of rats *in situ*. Staining of whole glands with X-gal revealed the location of infected cells. Adenovirus infected the end buds with high efficiency but failed to infect the primary/secondary ductal cells. Expression of lacZ from the adenoviral vector was transient, with no expression by day 8 post-infusion. Both amphotropic and pantropic lacZ retroviral vectors preferentially transduced the end buds, yet the pantropic vector was 45-fold more infectious than the amphotropic vector. Primary/secondary ductal cells were not transduced. For the pantropic lacZ vector, the percentage of transduced cells increased significantly from day 3 to day 10 post-infusion but sharply decreased by day 21. Staining also occurred in the pregnant gland post-infusion. To determine if retroviral expression of TGFβ-1 would prevent chemically-induced mammary carcinogenesis, rats were infused with an amphotropic TGFβ-1 retrovirus 1 or 3 weeks after treatment with the mammary carcinogen NMU. Control rats were infused with vehicle 3 weeks after NMU. Virally-infused rats showed no decrease in tumor incidence, tumors/rat, or tumor latency compared to control rats.

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INTRODUCTION

Breast cancer is the leading cause of cancer among women, striking 150,000 women per year, and 1 in 8 American women will develop breast cancer over the course of her lifetime. To date, little can be done in the way of breast cancer prevention. Prevention is limited to self-examination and frequent mammographies to detect early tumors. It is known that breast cancer is heritable in a small percentage of cases and knowledge of one's family history provides some relative risk level for developing breast cancer. In 1994, the BRCA1 gene was cloned and found to be associated with about 5% of breast cancer cases (1). Genetic tests are now available to screen women for a defective BRCA1 gene, thereby identifying women who are at high risk for developing breast cancer. It would then become ideal to have an effective breast cancer prevention strategy for these women. We therefore would like to determine if gene therapy technology can be used to prevent the development of breast cancer.

The two most common approaches in gene therapy protocols have relied on replication-defective retroviral and adenoviral vectors. Both of these viruses have inherent advantages and disadvantages as gene therapy vectors (reviewed in 2). Retroviral vectors can be packaged with various envelope proteins. A commonly used packaging protein is the murine amphotropic envelope protein. This allows the virus to infect many different cell types from a wide variety of species. However, the target cell must express the amphotropic receptor. Retroviral vectors may also be packaged with the vesicular stomatitis virus glycoprotein (VSV-G) (3). VSV-G allows the virus to infect cells through fusion with the plasma membrane (4). Because VSV-G does not require a specific protein receptor for viral entry, vectors packaged in this manner have pantropic tropism. Aside from the differences in the envelope protein, amphotropic and pantropic retroviruses are the same. Retroviruses (with the exception of the lentiviruses) require a target cell to be proliferating in order to integrate the viral cDNA into the host's genome (5). A technical limitation thus far in the use of retroviral vectors has been the difficulty in obtaining high titer viral stocks that are required for efficient therapeutic use *in vivo.*

Adenoviruses used in gene therapy procedures are also able to infect a large variety of cell types. Unlike the retroviruses, adenoviruses do not integrate their DNA into the host's genome as part of their normal replication lifestyle. Additionally, adenoviruses can readily infect nonreplicating cells as well as replicating cells (6). A disadvantage of adenoviral vectors is that expression of the therapeutic gene is usually transient due to the lack of integration. It has been demonstrated that a host immune response raised against adenoviral infected cells assists in vector clearance (7).

A technique well-established in our laboratory allows for the infusion of recombinant retroviruses directly into the rat mammary gland *in situ*. To date, lacZ (8), the ras gene (8), and the neu gene (9) have been successfully introduced into rat mammary epithelial cells (RMEC) using this technique. Therefore, we wish to investigate whether this technique of gene delivery to the rat mammary gland *in situ* can be used to introduce therapeutic genes into RMEC and thereby provide protection against the development of chemically-induced mammary carcinomas.

Retroviruses could be used to convey a negative growth regulating gene to RMEC. Stable long-term expression of a negative growth regulating gene would ideally keep the RMEC in permanent growth arrest. The cytokine transforming growth factor β-1 (TGFβ-1) is an appealing candidate molecule for a cytostatic gene therapy approach. TGFβ-1 is known to be a powerful negative growth regulator of most epithelial cells (reviewed in 10). *In vitro,* TGFβ-1 has been shown to be a negative growth regulator of the human breast cancer cell line MCF-7 (11). *In vivo,* Silberstein and Daniel demonstrated that implanted pellets releasing TGFβ-1 caused local growth inhibition of mammary end buds (12). A transgenic mouse for simian TGFβ-1 developed by Moses and colleagues exhibits mammary ductal hypoplasia (13). Follow up studies by the same laboratory showed that offspring of TGFβ-1 transgenic mice and TGFα transgenics that harbored both transgenes were resistant to mammary tumor formation, unlike their TGFα-only littermates (14). In addition, the TGFβ-1 transgenics were resistant to 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors (14). Thus, the available data suggests that TGFβ-1 might be a reasonable candidate molecule to produce a cytostatic effect on the mammary epithelia.

Due to the transient nature of adenoviral expression, adenoviruses would not be practical for a cytostatic gene therapy approach where long-term expression would be necessary. Instead, adenoviral vectors could be used for a cytotoxic gene therapy approach. In this approach, a gene would be chosen that could be delivered by an adenoviral vector and result in selective killing of the RMEC. This would have the desired result of obliterating the cellular origin of mammary carcinomas, theoretically removing the targets for chemical carcinogens. In essence, this could be considered an "epithelial mastectomy". Ideally, the mammary epithelium would be destroyed, but the stroma and outward appearance of the gland would not be affected. A category of genes that can be used for selective killing are the so called "suicide genes". These viral or prokaryotic genes encode for proteins that are lethal to mammalian cells only when presented with a specific drug, which they can activate to yield a metabolite lethal to the cell (reviewed in 15). Some suicide genes also kill cells indirectly through the "bystander effect". By this mechanism, the toxic metabolite passes freely between neighboring cells. This allows the toxicity to spread beyond the population of cells that express the suicide gene (16).

Another potential approach for initiating a cytotoxic effect against the mammary epithelium is to take advantage of the host's immune response

directed against virally-infected cells. It has been well-documented that infection with adenoviral vectors results in acute inflammatory responses directed by the host against the infected cells. This has been demonstrated in the lungs of baboons (17), mice (18), and cotton rats (19). In these studies, there was damage to the bronchial epithelium. This damage was initiated by the immune system in response to the expression of viral antigens by the infected cells. In addition, it could be possible to enhance this response through the use of genes that stimulate the immune system. These immune stimulating genes would be expressed in the adenoviral vector.

Before these various gene therapy approaches can be tested, it is necessary to determine the relative infection efficiencies of retroviral and adenoviral vectors in the rat mammary gland. This can be accomplished by assaying for the expression of a reporter gene carried by the viral vector. For our initial characterization of retroviral and adenoviral vectors, we are using the bacterial gene lacZ as a reporter gene in the viral vectors. These experiments with lacZ viral vectors constitutes the bulk of the work to be presented in this report. In addition, we also present data for our proposed cytostatic gene therapy approach using TGFβ-1 as the therapeutic gene.

BODY

METHODS

Construction of lacZ viral vectors

Previously in our laboratory (8), a lacZ retroviral producer clone was generated from the amphotropic retroviral packaging cell line PA317 (ATCC). This clone constitutively produces amphotropic packaged virus from the retroviral vector pJR/Gal (Amp-JR/Gal). Virus was harvested from conditioned media and concentrated by ultracentrifugation through a sucrose cushion as described (8). The titer of the concentrated virus was determined on NIH 3T3 cells (ATCC) and a helper virus assay was performed as described (20). A pantropic lacZ retroviral producer clone was constructed essentially as described (21). The cell line 293-gag/pol, a 293-derived cell line stably expressing Moloney Murine Leukemia Virus (MoMLV) gag and pol genes (provided by Viagene, Inc.), was infected with Amp-JR/Gal. Infection with Amp-JR/Gal yielded the stable cell line 293-gag/pol-JR/Gal. G418 resistant clones were selected by virtue of the neomycin phosphotransferase gene encoded by JR/Gal. Multiple individual clones were expanded and each stained with X-gal as described (20) to select the clone that contained the highest percentage of cells expressing β-galactosidase (β-gal). Clone 3 was selected for final virus production. To produce pantropic packaged virions (Pan-JR/Gal), 293-gag/pol-JR/Gal-3 line was transiently transfected by $CaPO_4$ with a plasmid (provided by Dr. Jiing Kuan-Yee) expressing VSV-G. Pan-JR/Gal containing media was collected 48-72 hr post-transfection. Pan-JR/Gal was concentrated, titered, and assayed for helper virus as described for Amp-JR/Gal. lacZ recombinant adenovirus (Ad5-lacZ) was provided by Dr. Jon Wolff (Waisman Center, Vector Core Laboratory, University of Wisconsin-Madison). Ad5-lacZ is $E1^-$ $E3^-$, with lacZ expression driven by the cytomegalovirus (CMV) promoter.

Infusion of viral vectors into the rat mammary gland *in situ*

The technique for the *in situ* delivery of retroviruses into the rat mammary gland has been developed in our laboratory (8). Rats were administered perphenazine, a mammary mitogen, at 3 mg/kg body weight dissolved in acidified saline (0.03 N HCl). Perphenazine was given by subcutaneous injection the day of the infusion and each of two days prior to the infusion. Rats were anesthetized with ether and the central duct of the gland was infused with 10-15 μl of retroviral vector stock (in DMEM + 10% FBS) containing 80 μg/ml polybrene and 3.0 mg/ml indigo carmine, a tracking dye to monitor the success of the infusion. This technique was also used with some modifications for the infusion of Ad5-lacZ. For adenovirus infusions,

rats were not pretreated with perphenazine. Adenoviral stocks were suspended in PBS and the viral solution infused did not contain polybrene but did contain indigo carmine at the same concentration as above. For all infusions, rats were 7 to 10-weeks of age at the time of infusion.

X-gal Staining of Rat Mammary Gland Whole Mounts

The protocol for the X-gal staining of rat mammary gland whole mounts was derived from (22) with modifications. Following infusion of lacZ viral vectors, the abdominal/inguinal fat pad was removed and mounted on a glass slide. The whole mounts were allowed to air-dry 5-10 min and then were placed in fixing solution (4% paraformaldehyde dissolved in PBS) for 2 hr at 4° C. After fixing, mounts were washed three times for 10 min in fresh washing solution (calcium, magnesium supplemented PBS [Gibco BRL] with 2.2 mM sodium deoxycholate [Boehringer Mannheim] and 0.18% Nonidet P40 [Boehringer Mannheim]). Mounts were then stained overnight at 37° C in fresh staining solution (calcium, magnesium supplemented PBS with 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM magnesium chloride, 2.2 mM sodium deoxycholate, 0.18% Nonidet P40 and 1 mg/ml X-gal in dimethylformamide). After staining, mounts were washed two times for 15 min in distilled water. Mounts were then defatted by successive immersions in 70% ethanol (2 x 1.5 hr), 95% ethanol (1.5 hr), 100% ethanol (overnight), chloroform:100% ethanol in a 1:1 ratio (30 min), and chloroform (2 x 30 min). After defatting, mounts were then rehydrated by successive immersions for 15 min each in 100% ethanol, 95% ethanol, and 70% ethanol. Mounts were stored in 70% ethanol for up to 2 weeks before microscopic analysis. Mounts were examined under 70% ethanol using an Olympus Stereomicroscope. Photomicrographs were taken using Kodak Ektachrome slide film, ISO 100.

Histological Preparations from X-gal Stained Rat Mammary Gland Whole Mounts

Areas of interest were microdissected. Tissue pieces (3-5 mm long) were dehydrated by successive immersions for 15 min each in 95% ethanol, 100% ethanol, chloroform:100% ethanol in a 1:1 ratio, and chloroform. Tissue was then transferred to fresh chloroform prior to being embedded in paraffin. 4 μm thick sections were cut from the embedded tissue and adhered to polylysine-coated slides. Samples were deparaffinized in chloroform (2 x 5 min) then rehydrated by successive immersions for 15 min each in 100% ethanol, 95% ethanol, 70% ethanol, and distilled water. Tissue was then stained for 10 min in 0.1% nuclear fast red (Poly Scientific). Slides were blotted dry and mounted with aqueous mounting media. Photomicrographs were taken using Kodak Ektachrome slide film, ISO 100 or 400.

RESULTS

in situ Localization of Ad5-lacZ Infected RMEC and Duration of lacZ Expression

Seven-week-old Wistar-Furth (WF) rats were infused with 10 µl of Ad5-lacZ at a titer of $6x10^9$ plaque forming units (PFU)/ml (n=8 rats) or vehicle control (n=1 rat). On days 2, 5, and 8 post-infusion, rats were sacrificed and mammary gland whole mounts were stained with X-gal. On day 2 (n=3 rats), a high percentage of the mammary end buds were stained blue, indicating that these cells were infected by Ad5-lacZ (Figure 1A). Glands from the vehicle control rat did not have any staining (Figure 1B). At a much lower frequency, mammary ducts that are directly connected to the end buds (tertiary ducts) were also stained (data not shown). Staining did not occur in the primary or secondary mammary ducts (Figure 1C). On day 5 (n=3 rats), staining was still observed in the same pattern (data not shown). However, the percentage of stained end buds was significantly reduced. By day 8 (n=2 rats), there was not any staining (data not shown). With the exception of blue staining, the gross appearance of the gland was normal for all of the time points. Histological analysis at day 5 showed the blue infected cells (end bud and tertiary ductal) among red non-infected cells (Figure 1D). The histological appearance of the gland was normal at both days 5 and 8. There were no obvious indications of inflammatory-induced damage to the mammary parenchyma.

Comparison of Amp-JR/Gal and Pan-JR/Gal Infection Efficiencies *in situ* and Localization of Infected RMEC

Eight-week-old WF rats were infused with 10 µl of Amp-JR/Gal (n=2 rats) or Pan-JR/Gal (n=2 rats). Each virus was at a titer of $4.4x10^7$ colony forming units (CFU)/ml. On day 2 post-infusion, rats were sacrificed and mammary gland whole mounts were stained with X-gal. For each group, staining was localized to the end buds (Figure 2A, 2D). Pan-JR/Gal also exhibited some staining in the tertiary mammary ducts (Figure 2C), but this occurred at a much lower frequency compared to end bud staining. Staining was not observed in the primary or secondary mammary ducts. Histological analysis indicated that Pan-JR/Gal was also capable of infecting myoepithelial cells (Figure 2E). To quantify the relative infection efficiencies between Amp-JR/Gal and Pan-JR/Gal, 3 mounts (equal to 6 glands) from each group were scored for the number of blue cells (Figure 3). Pan-JR/Gal was more infectious, with 45 fold more blue cells than Amp-JR/Gal.

Duration of lacZ Expression from Pan-JR/Gal *in situ*

Eight-week-old WF rats (n=10) were infused with 10 µl of Pan-JR/Gal at a titer of $5.8x10^7$ CFU/ml. On days 3, 10, and 21 post-infusion, 2 rats were sacrificed and mammary gland whole mounts were stained with X-gal. Relative to day 3, the number of blue cells on day 10 had increased dramatically. On day 10, there were numerous end buds that were almost completely blue (Figure 2B). Although there were highly stained buds also on day 3, the overall number was lower than on day 10. By day 21, the number of blue cells had fallen far below the day 3 level. There were only a few end buds with blue cells. For all three time points, staining was confined to end buds and, to a lesser extent, tertiary ducts. The number of blue cells/end bud ranged from one cell up to almost completely blue buds. This range of values for blue cells/end buds was observed at all three time points.

Effect of Mammary Gland Differentiation on lacZ Expression from Pan-JR/Gal *in situ*

Eight-week-old WF rats (n=16) were infused with 10 µl of Pan-JR/Gal at a titer of $5.8x10^7$ CFU/ml. Five-ten days post-infusion, 10 rats were mated to Spraque-Dawley males. The remaining 6 rats remained virgins. Five additional rats were used as negative controls. The negative controls were not infused, but were mated. On day 7 of pregnancy (day 12 post-infusion), 2 pregnant, infused rats and 1 pregnant, negative control rat were sacrificed and mammary gland whole mounts were stained with X-gal. One virgin, infused rat was also sacrificed at this time. Surprisingly, the virgin control glands were negative for staining. However, the pregnant infused glands were positive for end bud and tertiary ductal staining. As seen before, there was no staining in the primary or secondary ducts. Although the pattern of staining was identical to that of the virgin gland, the number of blue cells was lower than that seen previously on day 3 post-infusion in the virgin. On day 18 of pregnancy (day 27 post-infusion), 2 pregnant, infused rats, 1 pregnant, negative control rat, and 1 virgin, infused rat were sacrificed. There was no longer any staining in the virgin or the pregnant glands. For both pregnancy time points, the negative control glands did not stain.

Effect of TGFβ-1 on N-methyl-N-nitrosourea (NMU)-Induced Mammary Carcinogenesis

WF rats (n=74) at 49-55 days of age were given an intravenous dose of NMU at 50 mg/kg body weight. At one (n=24) or three (n=25) weeks post-NMU, the rats were infused in all 12 glands with 15 µl of Amp-JR/TGFβ-1 at $1.2x10^8$ CFU/ml. At three weeks post-NMU, a negative control group (n=25) was infused with 15 µl of vehicle in all 12 glands. Beginning at week 5 post-NMU, the rats were palpated weekly for the appearance of mammary tumors. The experiment was terminated at 19 weeks post-NMU. All rats were

necropsied and scored for tumor burden. The percent of tumor incidence was defined as the percentage of rats from each group with at least one mammary tumor. The total number of mammary tumors was divided by the number of rats from each group with tumors to yield tumors/rat. Latency was defined for each rat as the amount of time post-NMU until the first palpable tumor. The percentage of tumor incidence was 91.7, 88.0, and 92.0 respectively for 1-week infusion, 3-week infusion, and negative control. The average number of tumors/rat was 3.59, 2.73, and 2.87 respectively for 1-week infusion, 3-week infusion, and negative control. For all three groups, latency was 12-13 weeks. The data for this experiment is illustrated in figure 4.

DISCUSSION

Ad5-lacZ Does Not Infect the Primary or Secondary Ductal Cells

We proposed using adenoviral vectors for the prevention of breast cancer in the rat. The successful use of adenoviral vectors for this objective is dependent on three assumptions. First, that most of the RMEC will be infected following infusion of high titer adenovirus. Second, that the infected RMEC will be killed by an aggressive host immune response. Third, that RMEC killed will not be replaced through the replication of mammary stem cells that avoided infection.

In order to investigate the feasibility of using adenoviral vectors for this approach, we began by infusing Ad5-lacZ into the rat mammary gland. The objectives of this experiment was to determine which sub-population of RMEC would be infected by adenovirus and how long lacZ expression would last *in vivo*.

In the original proposal, we planned on addressing these issues by X-gal staining of cultured RMEC harvested from rats infused with Ad5-lacZ. Since then, we have established a technique for staining mammary gland whole mounts with X-gal *in situ*. This technique avoids any potential culturing artifacts and allows for the precise *in situ* localization of infected RMEC.

Adenovirus infection is known to be independent of the proliferative state of the target cell. In addition, adenovirus has the ability to infect a wide variety of cell types from several species. However, infection is dependent on the cellular expression of the appropriate receptors. Based on the broad tropism displayed by adenovirus, we expected that all sub-populations of RMEC would be infected by Ad5-lacZ. In order to confirm this, Ad5-lacZ was infused and mammary gland whole mounts were collected on days 2, 5, and 8 post-infusion and stained with X-gal. Indeed, Ad5-lacZ infected the end buds with a very high efficiency (Figure 1A). To a lesser extent, tertiary ducts were also infected. Unexpectedly, neither the primary or the secondary ducts were infected (Figure 1C). These results contrast with those of Nandi (23). Nandi and colleagues infused mice with Ad5-lacZ. Although they did not

completely characterize the *in situ* infection, their results show infection of the mammary ducts. The infused adenovirus must traverse the ducts in order to reach the end buds. Because the end buds were readily infected, our results suggest that the rat mammary ductal cells do not efficiently express the adenovirus receptor, thereby avoiding infection. Our results and those of Nandi suggest that mice and rats show differential expression of the adenovirus receptor on the mammary ductal cells.

Numerous investigators have shown that the duration of expression from recombinant genes in adenoviral vectors is transient *in vivo.* In our original proposal, we demonstrated by X-gal staining of cultured RMEC that expression of lacZ at day 10 post-infusion occurs in less than 1% of the RMEC. In the current experiment, we reproduced the transient duration of expression. The percentage of stained cells decreased significantly from day 2 to day 5. By day 8, no staining was detected.

It has been demonstrated elsewhere that the transient nature of recombinant adenoviral expression is due in large part to an aggressive host immune response directed at infected cells (7). In this experiment, we did not formally investigate the immune response in the mammary gland following Ad5-lacZ infusion. However, we did examine histological sections of infected areas. Unfortunately, sections from day 2 were ruined during processing. At this time, the methodology for processing the sections was still in the experimental stages. Sections for day 5 and day 8, however, were obtained. Histologically, the appearance of the gland at both time points was normal. Since expression of lacZ declined significantly from day 2 to day 5, it was expected that there would be histological indications of inflammatory-induced damage to the mammary parenchyma at day 5. However, this did not seem to be the case (Figure 1D). Although this experiment demonstrated that recombinant adenoviral expression is indeed transient in the infected rat mammary gland, it cannot be concluded how this decrease in expression came about. Since adenovirus does not integrate its DNA into the host's chromosomes, it is possible that the loss of adenoviral DNA from the infected RMEC contributed to the decline in expression. If the decrease in expression was due in part to an immune response directed at the infected RMEC, then this response did not leave obvious histological indications. Considering that the histological appearance of the gland was normal at day 8 post-infusion, the mammary gland might be capable of quickly regenerating epithelium that was destroyed by an immune response.

This experiment demonstrated that the first assumption made for adenovirus infection of the mammary gland was not valid. The primary/secondary mammary ductal cells were not infected by Ad5-lacZ. This has important implications for gene therapy applications. My proposed experiments required that all RMEC be infected to a high degree. This is necessary if most of the mammary parenchyma is to be permanently ablated in a cytotoxic gene therapy approach. However, since the ductal cells escape infection by adenovirus, they would never be targeted by the immune response. Another cytoxic gene therapy approach proposed the use of suicide

genes to directly kill adenovirus infected cells and to indirectly kill through the bystander effect. Again, the ductal cells would escape the direct killing by virtue of escaping adenovirus infection. Considering that the ductal cells are physically removed from the infected end bud cells, it is unreasonable to expect the bystander effect to have any significance in the indirect killing of the ductal cells. In summary, the possibility of permanently ablating the rat mammary epithelium seems unrealistic in light of the results of this experiment. No matter which cytotoxic approach was employed, the ductal cells would not be infected by the adenoviral vector and would therefore not be killed. Thus, the goal of total mammary epithelium ablation would not be realized. Any putative mammary stem cells located in the ducts would have the potential to repopulate the gland, thereby replacing end bud cells that were killed by adenovirus infection. Even if repopulation of the gland did not occur, the remaining ductal cells would have the potential to develop into ductal carcinomas upon challenge with carcinogen. Therefore, the cytotoxic gene therapy approaches originally proposed no longer seem practical.

Pan-JR/Gal is More Infectious Than Amp-JR/Gal and Both Pseudotypes Preferentially Transduce End Bud RMEC

We proposed the use of retroviral vectors to convey cytostatic genes to RMEC as an additional gene therapy approach. We wished to determine both the *in situ* localization of infected RMEC and the relative infection efficiencies between amphotropic and pantropic retroviral vectors in the rat mammary gland.

To address these issues, we used lacZ retroviral vectors packaged either with amphotropic or pantropic pseudotypes. Following infusion of lacZ retroviral vectors, mammary gland whole mounts were stained with X-gal. Both viruses stably transduced end buds predominantly (Figure 2). In addition, Pan-JR/Gal also transduced tertiary ducts, but at a much lower frequency. Significantly, the primary and secondary ducts were not transduced by either retroviral pseudotype. A quantitative comparison between the two pseudotypes showed that Pan-JR/Gal was more infectious than Amp-JR/Gal by 45-fold. The only difference between the two retroviruses is the envelope protein used to package the virus. Amp-JR/Gal uses the amphotropic envelope protein for packaging and the target cells must contain the amphotropic receptor. In contrast, Pan-JR/Gal uses the VSV-G protein for packaging. VSV-G allows the virus to enter cells through fusion with the plasma membrane. Therefore, it is not necessary for the target cell to be expressing a specific protein receptor. Because Amp-JR/Gal and Pan-JR/Gal were infused at equal titers, but the latter infected more cells, it is possible that only a small fraction of RMEC express the amphotropic receptor. Thus, Pan-JR/Gal presumably infects RMEC that are resistant to Amp-JR/Gal infection.

Although Amp-JR/Gal and Pan-JR/Gal show a difference in relative infectivity, both retroviruses require proliferating cells for stable integration of the proviral DNA. Our results therefore demonstrate that the end buds and some of the tertiary ducts contain the proliferating RMEC at the time of infusion. This in in agreement with the observations of Russo (reviewed in 24). It must be emphasized, however, that even Pan-JR/Gal was capable of transducing only a small fraction of the total number of end buds within the gland. The requirement of proliferating cells for retroviral transduction therefore severly limits the number of RMEC that can be transduced by either retroviral vector.

Both viruses fail to transduce the primary and secondary ductal cells but our findings cannot rule out the possibility that one or both of the retroviruses are actually infecting the ductal cells. If these cells were proliferating, than it could be assumed that at least Pan-JR/Gal would transduce these cells since a specific protein receptor would not be needed for infection. Our results suggest that the ductal cells are not proliferating and therefore are not stably transduced by either retroviral vector.

Duration of lacZ Expression from Pan-JR/Gal *in situ*

In order to determine the duration of lacZ expression following infusion, whole mounts were removed and stained with X-gal at various time points following infusion. The author chose to do this experiment only with Pan-JR/Gal due to its higher infection efficiency. At day 10 post-infusion, the number of stained cells had increased dramatically relative to day 3. Notably, there were many end buds containing numerous blue cells (Figue 2B). By day 21, however, the number of stained cells had decreased to well below the level seen at day 3. At all three time points, there were end buds containing different numbers of stained cells. These values ranged from one cell to almost complete staining of the bud. This experiment is ongoing, with time points at 3 months and 6 months still to come. Very recently, another individual in the laboratory stained whole mounts 1.5 years after Amp-JR/Gal infusion. Surprisingly, there was still staining (data not shown). Earlier timepoints, however, were not investigated.

Our results from this experiment thus far suggest that Pan-JR/Gal infects a sub-population of end bud cells that are capable of multiple rounds of proliferation. This could explain why at day 10 post-infusion there were numerous buds almost completely stained. This sub-population could possibly be mammary stem cells. However, it is unlikely that all of the transduced cells have stem cell-like characteristics. Since the number of stained cells decreased significantly by day 21, the majority of transduced cells presumably are not capable of continual proliferation and thus die off. In addition, at day 21, there were still end buds with only one stained cell. As this experiment is still ongoing, it cannot be concluded how long lacZ from Pan-JR/Gal will be expressed *in vivo*. In light of the very recent results, it can be stated that lacZ from Amp-JR/Gal can be expressed up to 1.5 years after

infusion. It remains to be determined if cells transduced by Pan-JR/Gal will show a similar long-term lacZ expression.

Effect of Mammary Gland Differentiation on lacZ Expression from Pan-JR/Gal *in situ*

To further characterize the nature of cells transduced by Pan-JR/Gal, we mated rats after infusion in order to determine if lacZ would still be expressed in the differentiated gland. At day 7 of pregnancy (day 12 post-infusion), staining still occurred predominantly in the end buds. To a lesser extent, there was staining in the tertiary ducts and myoepithelial cells. However, the number of stained cells was lower than that previously seen on day 3 post-infusion. At day 18 of pregnancy (day 27 post-infusion), no staining was observed. This experiment is still ongoing. Glands will also be stained in the lactating and regressing states. It is too early to draw many conclusions from this experiment. At the present time, it can only be said that the tranduced end bud cells are at least capable of undergoing partial differentiation.

Retroviral Expression of TGFβ-1 Does Not Prevent NMU-Induced Mammary Tumors

We proposed the use of a retroviral vector to deliver TGFβ-1 into RMEC via ductal infusion. We hypothesized that overexpression of TGFβ-1 would growth-arrest the infected cells, thereby preventing NMU-induced carcinogenesis. In our proposal, we described the construction of Amp-JR/TGFβ-1 and demonstrated that TGFβ-1 encoded by this virus is biologically active (Proposal, Figure 2). Amp-JR/TGFβ-1 was infused at either one or three weeks post-NMU. As illustrated in Figure 4, infusion of Amp-JR/TGFβ-1 did not prevent NMU-induced mammary tumors. Similarly, infusion of Amp-JR/TGFβ-1 did not decrease tumor latency. It must be reported that the latency data presented is probably an overestimation of the actual time required to develop the first tumor. For each group, there were some rats palpated as negative but found to have small tumors upon necropsy. In this category, there were 6 rats for 1-week infusion, 6 rats for 3-week infusion, and 7 rats for the negative control. Rats in this category were excluded from the latency data.

In our proposal, we stated that if pantropic vectors proved to be more infectious than amphotropic vectors, then this experiment would be done with a pantropic TGFβ-1 vector. However, this experiment was actually done with Amp-JR/TGFβ-1. This experiment was begun before the pantropic packaging system was fully operational in our laboratory. We feel, however, that the use of a pantropic vector in this experiment still would not have prevented mammary tumors. Despite the greater infectivity of pantropic vectors, the total number of RMEC infected is still only a small fraction of the total cell number within the gland. The number of potential NMU target cells would still be far greater than the number of infected cells. Additionally,

with the discovery that the retroviral vectors only transduce end bud cells, it would not be possible to prevent the formation of carcinomas arising from ductal cells by using retroviral vectors.

Although we did not formally test for the expression of TGFβ-1 *in vivo,* we assume that in fact TGFβ-1 was being expessed from the vector. We assume this based on the fact that TGFβ-1 was demonstrated to be expressed from the vector *in vitro.* Additionally, it is known that the retroviral vector JR functions *in vivo.* From data generated in this laboratory, there is a perfect correlation between *in vitro* and *in vivo* expression for the genes lacZ, ras, and neu.

CONCLUSION

In order to determine the feasibility of using gene therapy approaches for the prevention of breast cancer, we have performed initial experiments using lacZ recombinant viral vectors. Our purpose in using lacZ as a reporter gene was to determine the relative infection efficiencies of adenoviral and retroviral vectors and to determine how long recombinant genes are expressed in these viral vectors. Since our original proposal, a technique was established that allows for X-gal staining of infected rat mammary glands *in situ*. Using this new technical tool, we were able to determine the specific sub-population of mammary cells infected by each of the viral vectors.

We have found that adenovirus infects the mammary end buds with high efficiency but fails to infect the main ductal cells. Expression of lacZ *in vivo* from the adenoviral vector was transient, with no expression detected 8 days after infusion. Histological analysis at days 5 and 8 post-infusion did not show any obvious signs of inflammatory-induced damage to the epithelium, with the mammary gland having a normal histological appearance. However, our results cannot rule out the possible role of the immune response in vector clearance.

Our results show that it would be impractical to infect ductal cells and, subsequently, kill them with adenoviral vectors. Our proposed experiments relied on the assumption that all sub-populations of RMEC would be infected by adenovirus and thus killed by the immune response and/or cytotoxic genes expressed by the vector. If a large percentage of cells survived following infection, then there would be the potential for the surviving cells to repopulate the mammary epithelium. Therefore, these results strongly indicate that our proposed cytotoxic gene therapy approaches would not succeed.

In another set of experiments, we compared the relative infection efficiencies of amphotropic and pantropic packaged retroviral vectors. Using Pan-JR/Gal, we also examined the duration of lacZ expression after infection and the effect of mammary gland differentiation on the infected cells.

We found that both retroviral pseudotypes preferentially transduce the end bud cells. We have never found the main ductal cells to be transduced by either pseudotype. This observation is significant, as it indicates that retroviral vectors can be used to selectively label end buds. We also found that pantropic vectors are 45 fold more infectious than amphotropic vectors. But despite the greater infectivity of the pantropic vectors, only a small percentage of the total number of end buds were transduced.

Our experiments examining duration of lacZ expression and the effects of differentiation on the infected cells are still ongoing, so it is too early to draw definitive conclusions. To date, we have found that the percentage of transduced cells increases significantly by day 10 post-infusion but decreases sharply by day 21. Very recent results in our laboratory show that RMEC

infected by Amp-JR/Gal are still expressing lacZ 1.5 years after infusion, which means that the expression of the recombinant gene from the retroviral vector is extremely stable *in vivo*. It remains to be determined if RMEC infected by Pan-JR/Gal will show similar recombinant gene stability. Our results thus far do suggest that a proportion of RMEC transduced by Pan-JR/Gal are capable of further rounds of cellular proliferation, resulting in an increased percentage of stained cells at day 10. These results indicate that we are potentially infecting mammary stem cells with retroviral vectors. We are currently investigating this possibility.

The cells transduced by Pan-JR/Gal are capable of at least some differentiation. Glands stained at day 7 of pregnancy contained end buds with blue cells. However, there was not any staining at day 18 of pregnancy. It is too early to draw any further conclusions from this experiment.

To investigate the potential of TGFβ-1 to inhibit mammary carcinogenesis, we infused Amp-JR/TGFβ-1 one or three weeks after administration of NMU. Retroviral expression of TGFβ-1 did not prevent the development of NMU-induced mammary tumors. There was no decrease in % of tumor incidence, tumors/rat, or tumor latency. Therefore, our proposed use of TGFβ-1 for a cytostatic gene therapy approach was unsuccessful.

In this report, we present evidence to strongly suggest that our proposed gene therapy approaches would not be successful. In the interest of developing a doctoral thesis, it was decided by my major professor and thesis committee that a different project was necessary. Since our original proposal was based on the use of viral vectors, it was decided that the new project would also encompass the use of retroviral vectors to take advantage of the techniques and the knowledge already obtained. Please find a different proposal and modified statement of work in the Appendices.

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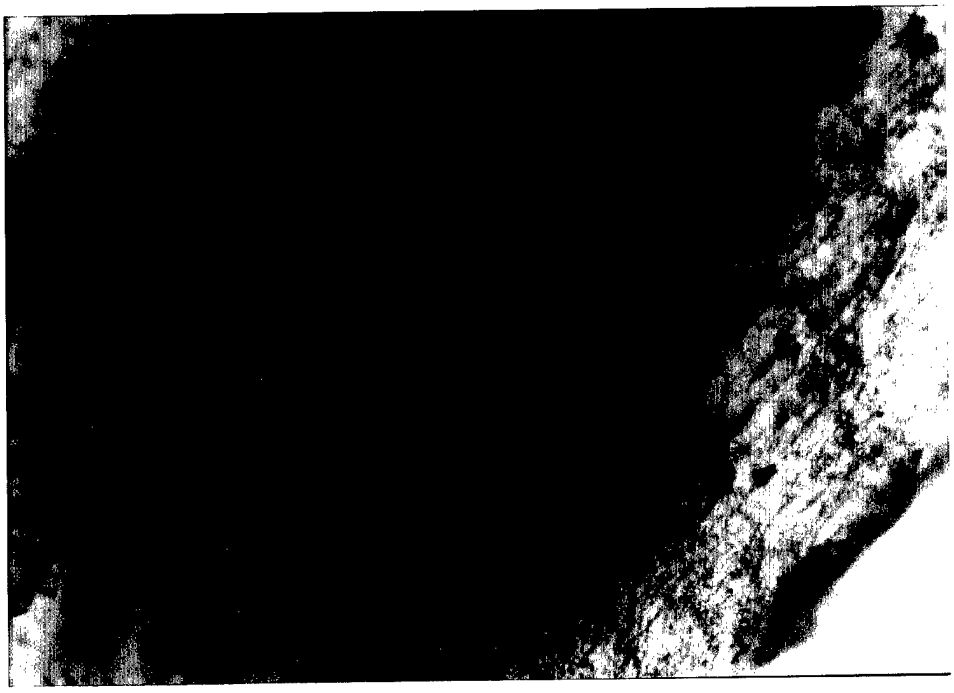
APPENDICES

FIGURES



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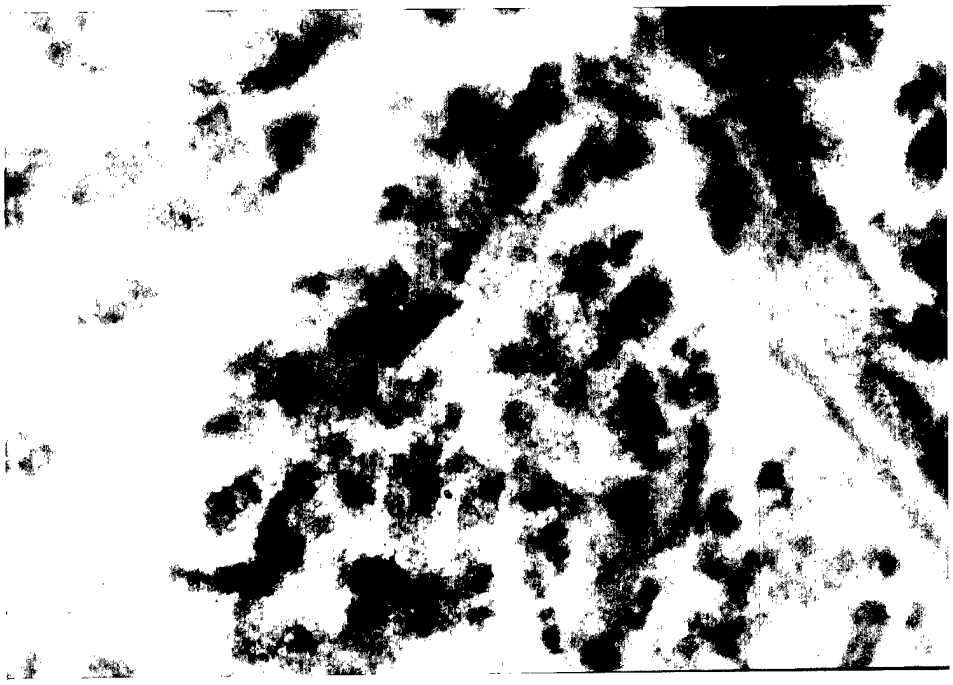

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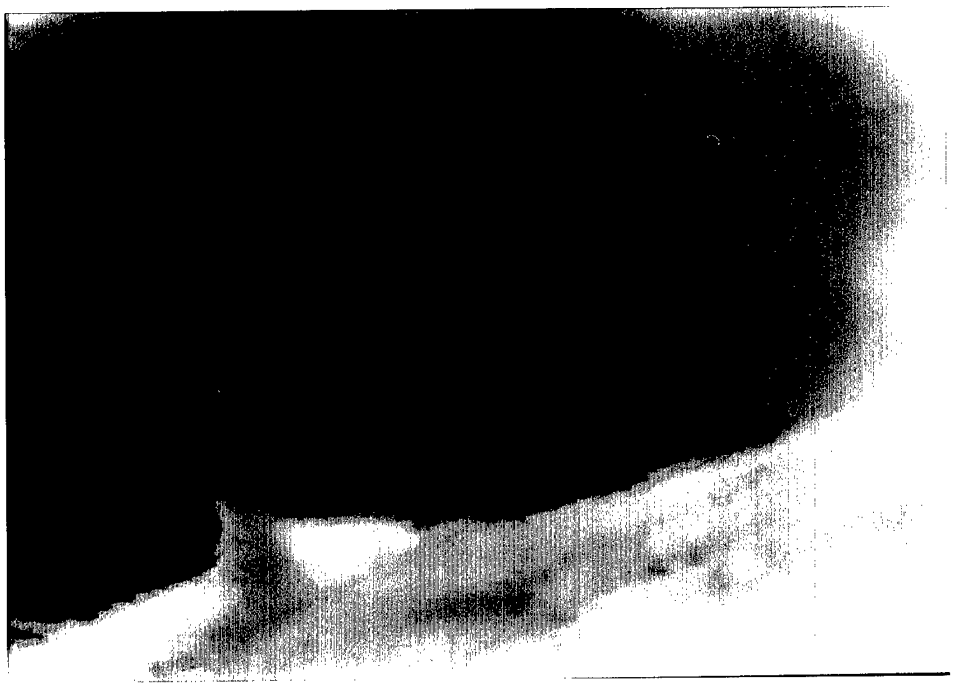

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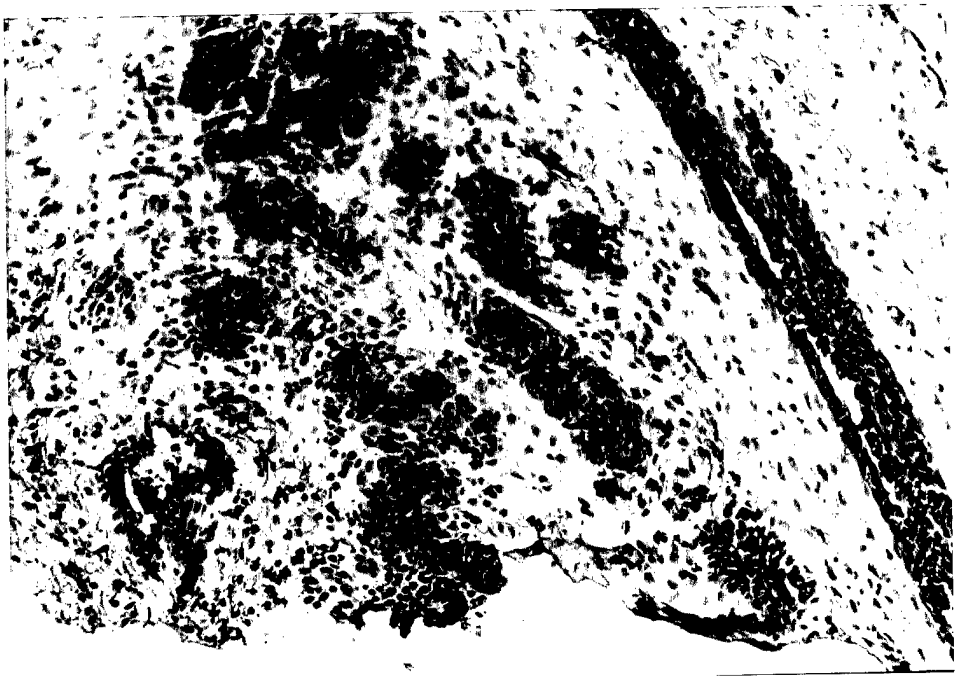

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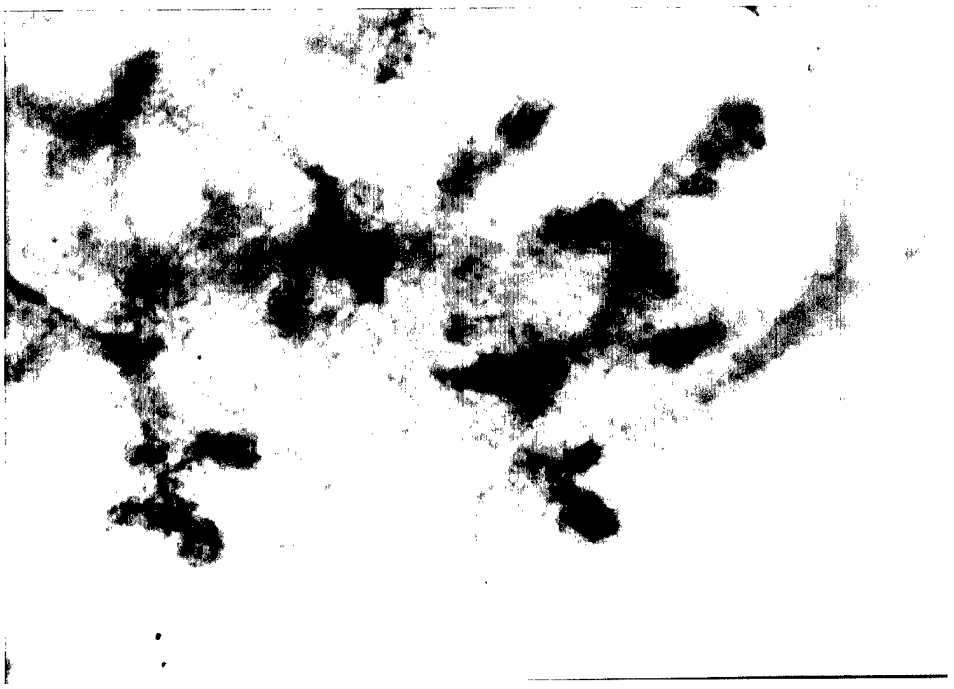

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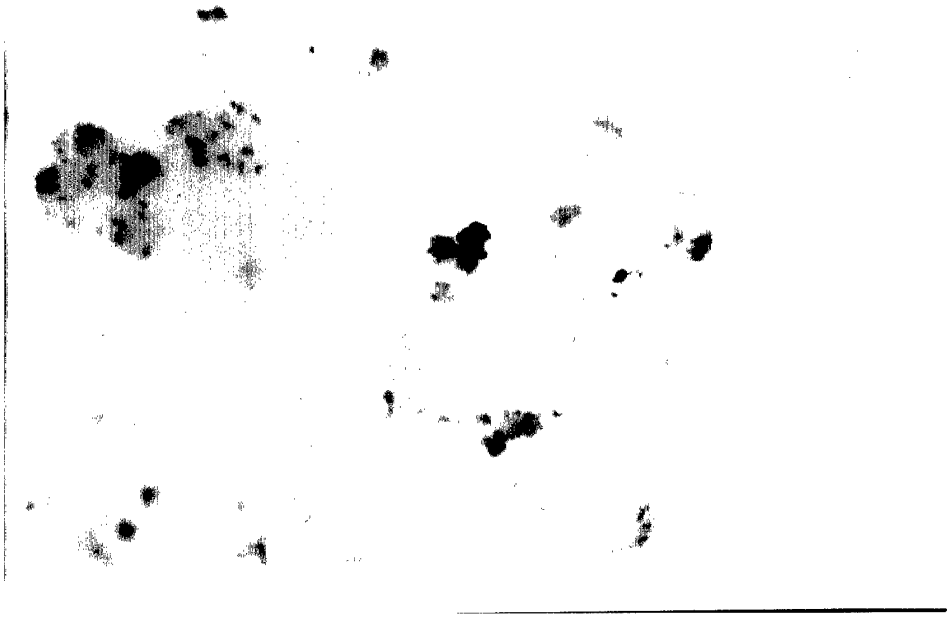

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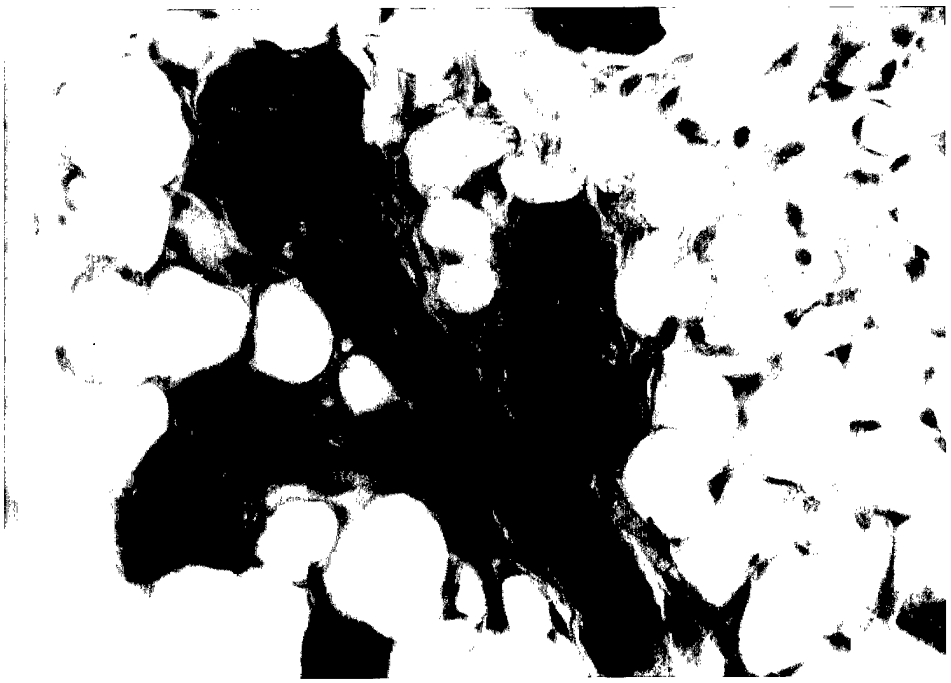

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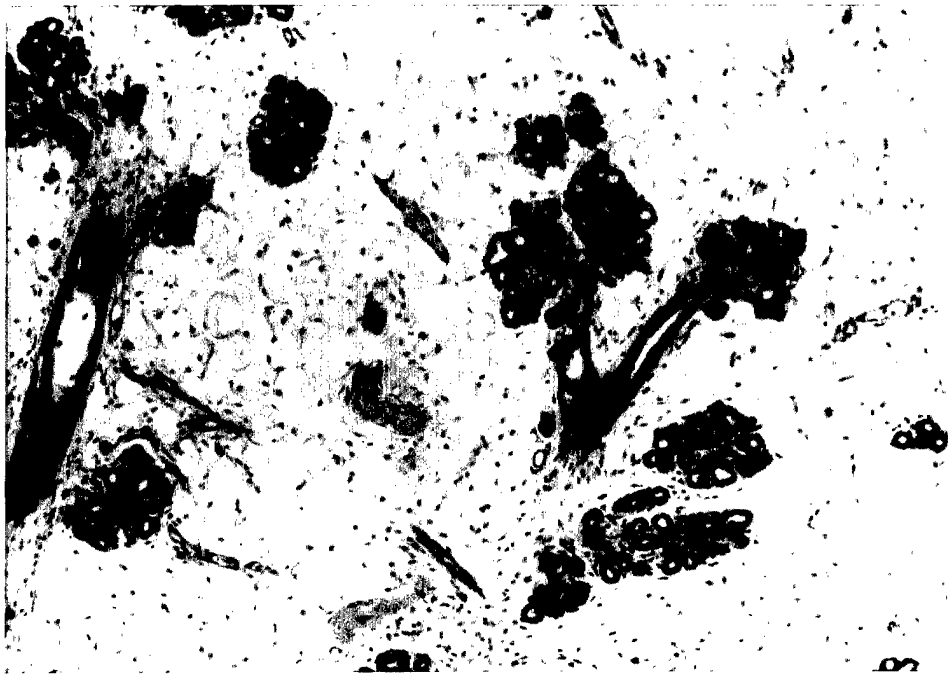

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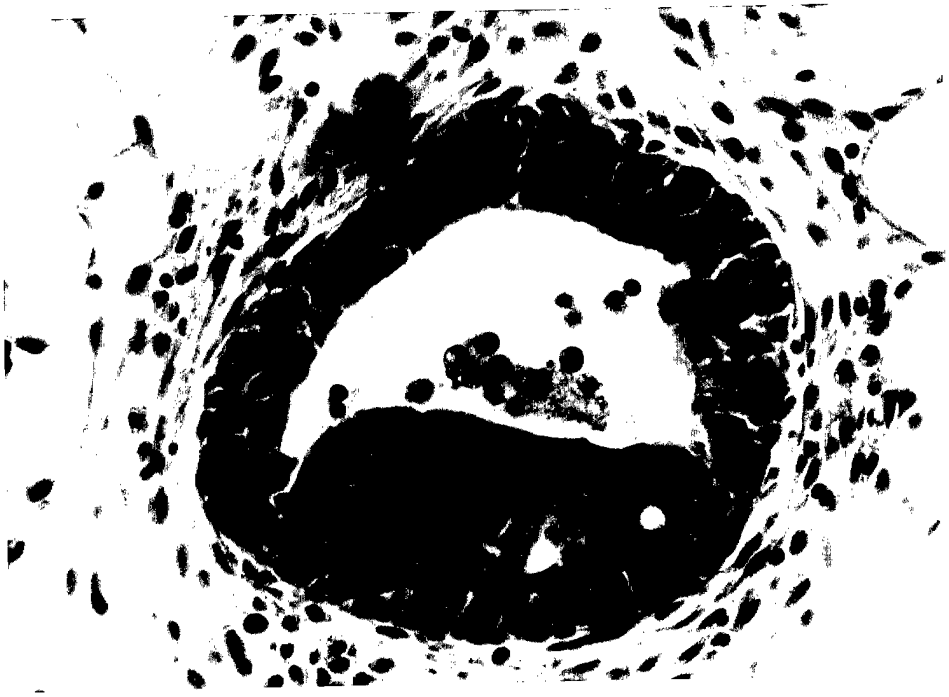

2E

FIGURE 1 AND FIGURE 2 LEGENDS

1A Whole mount mammary gland stained with X-gal 2 days post-infusion of Ad5-lacZ. The white strip running from up/down is the primary mammary duct. 60X.

1B Whole mount mammry gland stained with X-gal 2 days post-infusion of PBS. 40X.

1C Whole mount mammary gland stained with X-gal 2 days post-infusion of Ad5-lacZ. The brown mass is the inguinal lymph node. Stained end buds are above the lymph node. The white strip running from right/left below the lymph node is the primary mammary duct. 40X.

1D Histology of mammary gland 5 days post-infusion of Ad5-lacZ. At the right of the photo is a tertiary mammary duct. 200X.

2A Whole mount mammary gland stained with X-gal 3 days post-infusion of Pan-JR/Gal. This photo is from the terminal end buds of the abdominal gland. 70X.

2B Whole mount mammary gland stained with X-gal 10 days post-infusion of Pan-JR/Gal. 70X.

2C Histology of mammary gland 12 days post-infusion of Pan-JR/Gal. This rat was in day 7 of pregnancy at the time of staining. A blue tertiary ductal cell is represented in this photo. 400X.

2D Histology of mammary gland 10 days post-infusion of Pan-JR/Gal. 100X.

2E Histology of mammary gland 3 days post-infusion of Pan-JR/Gal. This section is from the terminal end buds of the abdominal gland. Blue myoepithelial cells are represented in this photo. 400X.

	Amp-JR/Gal	Pan-JR/Gal
Mount 1	22	751
Mount 2	28	963
Mount 3	14	1113
Mean	21	942

Figure 3: Amp-JR/Gal and Pan-JR/Gal Infection Efficiencies *in situ*

For each pseudotype, 3 mounts were scored. Values represent the number of blue cells/mount. The mean value of Pan-JR/Gal is 44.9 fold higher than the mean value of Amp-JR/Gal.

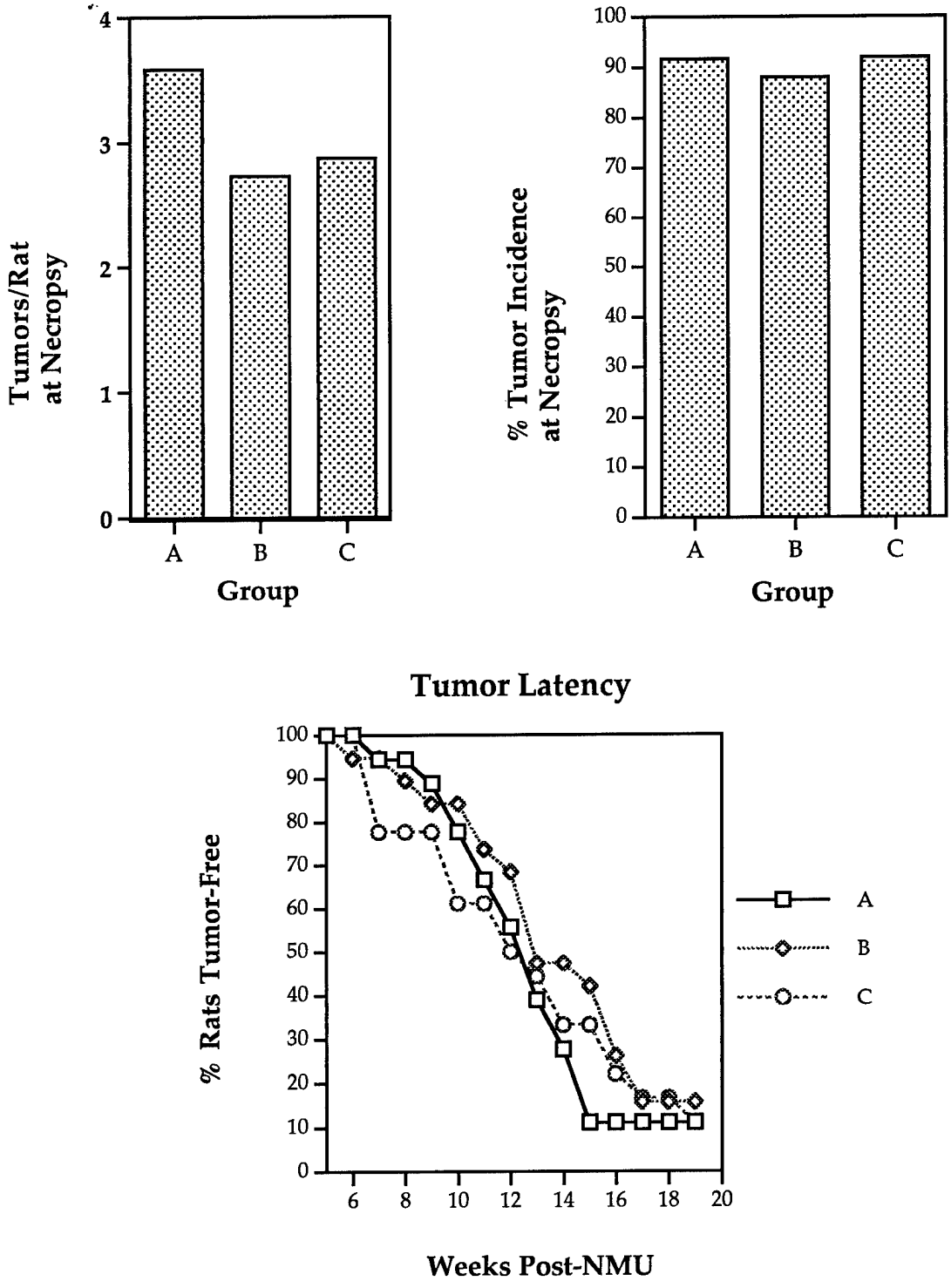

Figure 4: Effect of TGFβ-1 on NMU-Induced Carcinogenesis

Group A-infusion of Amp-JR/ TGF β-1 1 week post-NMU
Group B-infusion of Amp-JR/ TGFβ-1 3 weeks post-NMU
Group C-infusion of vehicle control 3 weeks post-NMU

Investigating the Role of Cooperative Interactions Between the neu Protooncogene and the Other erbB Family Members in Rat Mammary Carcinogenesis

BODY OF PROPOSAL

BACKGROUND

Tyrosine kinase growth factor receptors make up an important class of proteins involved in the growth and differentiation of eukaryotic cells. The epidermal growth factor receptor (EGFR) became the first member of the type I family of tyrosine kinase growth factor receptors. Since then, three other proteins have been added to this family; HER2/erbB-2/neu, HER3/erbB-3, and HER4/erbB-4. (For the remainder of this proposal, the erbB nomenclature will be used to name the proteins). All four members of this family are approximately 180 kD single-chain transmembrane spanning proteins composed of an extracellular ligand binding domain, a cytoplasmic tyrosine kinase domain, and a cytoplasmic C-terminus. erbB-2/neu, erbB-3, and erbB-4 share significant homology to EGFR. In particular, the tyrosine kinase domains are highly conserved among the four members while the C-terminus shows the lowest degree of homology (reviewed in 1).

Aberrant overexpression of the erbB receptors has been reported to be associated with several human malignancies (2-5). In particular, overexpression of EGFR and erbB-2/neu is found in a large percentage of human breast cancers. This overexpression is generally correlated with a poor patient prognosis (6). Additionally, erbB-3 overexpression has also been associated with some human breast cancers, but this relationship for erbB-3 is not as straightforward as for EGFR and erbB-2/neu in terms of patient prognosis (7).

Although overexpression of erbB-2/neu in human breast cancers is found in a large percentage of cases, the exact role that erbB-2/neu plays in the etiology of human breast cancer is unknown. In order to investigate the role of erbB-2/neu overexpression in mammary gland carcinogenesis, Muller and colleagues generated mice transgenic for the neu protooncogene (neu N) under the control of the mouse mammary tumor virus (MMTV) promoter (8). These mice developed mammary tumors with a long latency and the tumors were associated with overexpression of neu. Additionally, these tumors showed increased neu tyrosine kinase activity. However, follow up studies revealed that the majority of tumors in these transgenic mice were derived from somatic mutations in the neu transgene (9). These mutations resulted in the constitutive dimerization of neu, leading to the elevated neu tyrosine kinase activity (10). In human breast tumors, however, activating mutations in erbB-2/neu have not been detected. Therefore, these studies with the transgenic mice cannot be exactly correlated with the role of neu N in human breast carcinogenesis.

Three years ago, our laboratory generated transgenic rats with the MMTV-neu N construct created by Muller. It was expected that the transgenic rats would also develop mammary tumors. However, in two transgenic lines generated, neu-induced mammary tumors did not arise (unpublished

observations). Because Muller's transgenic mice developed somatic mutations in the neu N transgene, this raised two possibilities. One, that similar mutations did not arise in the transgenic rats. Alternatively, that these mutations did occur but are not oncogenic in the rat. To address the second possibility, two mutated neu cDNAs described in (9) were cloned into our retroviral vector JR (11) and infused into rats. Both neu mutants were highly oncogenic, with the entire mammary epithelium transformed by 6 weeks post-infusion (unpublished observations). Therefore, the neu mutations described by Muller are, in fact, oncogenic in the rat. However, the possibility still remains that the rats do not develop somatic mutations in the neu N transgene. Our observations demonstrate that neu N transgenic mice and rats have different phenotypes despite being generated from the same construct. Because human breast tumors have not been found to contain mutated neu, our transgenic rat is a potentially useful experimental model for the role of neu overexpression in human breast cancer.

The activation of type I growth factor receptors is dependent on receptor dimerization. The mechanism for dimerization was first deduced for EGFR, the prototypical member of this gene family (reviewed in 1). Upon ligand binding, EGFR dimerizes and this activates the tyrosine kinase activity. EGFR then undergoes auto-phosphorylation in the cytoplasmic domain. Phosphorylated tyrosines are then recognized by adaptor signaling molecules with SH2 domains. The activated EGFR is also capable of phosphorylating other proteins in the signal transduction pathways. Similarly, erbB-2/neu also requires dimerization for activation of the tyrosine kinase activity. As mentioned above, mutations exist in neu that turn the protooncogene into an active oncogene. The original oncogenic form of neu was cloned from neuroblastomas generated in rats treated with carcinogen (12). This mutant consists of a single amino acid change in the transmembrane domain. The mutation allows the protein to exist primarily as a dimer, while neu N remains a monomer (13). The end result of constitutive dimerization is an increased tyrosine kinase activity and cellular transformation (12, 14). Indeed, the activated neu mutant is an extremely potent oncogene *in vivo*. This has been demonstrated both in transgenic mice (15, 16) and in rats with our own retroviral infusion model (17).

Although erbB-2/neu can exist as an activated oncogene, there is at present no such activated forms of EGFR, erbB-3, or erbB-4. For these proteins, activation of the tyrosine kinase activity is dependent on ligand-induced dimerization. An interesting characteristic of the type I growth factor receptors is their ability to form heterodimers with other members of the family (reviewed in 1). Specifically, erbB-2/neu can form heterodimers with each of the other three members of the family and appears to be their preferred heterodimer partner (18). erbB-2/neu is an orphan receptor, with no known ligand. However, the ability of erbB-2/neu to form heterodimers allows for signaling by various ligands when the ligand is bound to the heterodimer partner (reviewed in 1). Specifically, a EGFR:erbB-2/neu heterodimer is responsive to ligands of the epidermal growth

factor/transforming growth factor α (EGF/TGFα) family which bind to EGFR. Likewise, erbB-2/neu heterodimers with erbB-3 or erbB-4 are responsive to ligands of the heregulin/neu differentiation factor (NDF) family. This complex network of heterodimer and homodimer formation among members of the family potentially regulate numerous growth and differentiation signal transduction pathways. Which signal transduction pathway is activated in a particular cell would be determined by a number of factors. These include the type and quantity of ligand available to the cell and which type I growth factor receptors are being expressed and their relative quantity.

Certain heterodimer combinations result in an increased transforming potential in NIH 3T3 cells. Specifically, erbB-2/neu was transforming when coexpressed with erbB-3 or EGFR (19). In mice doubly transgenic for neu N and TGFα, there is a synergistic interaction between the two genes that lead to a decreased tumor latency. The authors suggest that this synergy arises from a EGFR:neu N interaction (20).

Based on these findings, it seems plausible that neu N in our transgenic rats would be capable of forming heterodimers with the other members of the protein family if they were co-overexpressed. Since the expression of neu N in our transgenic rats is insufficient for mammary carcinogenesis, it is tempting to speculate that the co-overexpression of some or all of the other family members will lead to a synergistic interaction for mammary tumorigenesis. Investigating the potential cooperativity of neu N and the other family members in our transgenic rat model could help to delineate the role that erbB-2/neu overexpression plays in the etiology and progression of human breast cancer.

HYPOTHESIS

We hypothesize that overexpression of neu N in the transgenic rat fails to transform the mammary epithelium because of low neu tyrosine kinase activity. We predict that this is due to the presence of neu primarily as a monomer, instead of the activating dimer. We hypothesize that retroviral co-overexpression of other members of the erbB family in this transgenic strain will lead to heterodimer formation with neu. We would expect that such heterodimers will lead to elevatated neu tyrosine kinase activity in response to endogenous ligands. Lastly, we hypothesize that co-overexpression of some or all of the other erbB family members will lead to the generation of mammary carcinomas in the neu N transgenic rat.

OBJECTIVES AND EXPERIMENTAL APPROACH

Objective 1: We will generate additional lines of neu N transgenic rats. We will confirm that all lines have the same phenotype as previously observed. If the phenotype is duplicated in the additional lines, then one line will be chosen for further experimentation. If it is possible to maintain homozygosity in the chosen line, further experiments will be done with homozygous rats.

Objective 2: We will determine if neu N predominantly exists as a monomer or as a dimer in the transgenic rat. This will be done by native SDS-PAGE and Western blotting of neu isolated from transgenic mammary gland. We will determine the intrinsic tyrosine kinase activity of neu N by immunoprecipitating neu, followed by *in vitro* tyrosine kinase assays.

Objective 3: We will generate retroviral constructs for EGFR, erbB-3, and erbB-4. The cDNAs for these genes have been provided to us already. These genes will be cloned into a retroviral vector under the control of the long terminal repeat (LTR) promoter. This vector uses green fluorescent protein (GFP) as a selectable marker under the control of an internal promoter. This GFP based vector (LCG) was generated in this laboratory. The vector LCG has been successfully infused into rats and this renders the infected RMEC fluorescent when excited by blue light. Additionally, LCG-infected RMEC have been successfully collected by a Flow Cytometric Cell Sorter.

Objective 4: Each of the retroviral constructs generated under objective 3 will be infused into neu N transgenic rats. The rats will be palpated for the appearance of mammary tumors. If tumors are generated from any of the retroviral constructs, then we will attempt to determine if neu formed heterodimers. This will be done by immunoprecipitation of neu followed by Western blotting for the putative heterodimer partner. If heterodimers are found, we will determine the tyrosine kinase activity of the dimer.

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STATEMENT OF WORK

For the time period: Years 2-4

Objective 1

Task 1: Generation of additional lines of MMTV-neu N transgenic rats by pronuclear injection (being done by the University of Wisconsin-Madison Transgenic Facility). Months 13-19.

Task 2: Determining the tumorigenic phenotype of additional transgenic lines. Months 15-20.

Task 3: Generation of homozygous transgenic line. Months 15-20.

Objective 2

Task 1: Determining dimerization status and tyrosine kinase activity of neu N from the transgenic rat. Months 21-23.

Objective 3

Task 1: Construction of retroviral constructs and production of concentrated virus. Months 23-28.

Objective 4

Task 1: Infusion of each erbB retrovirus for tumorigenesis experiment. Each experiment will be carried out to 6 months. Months 29-34.

Task 2: Characterization of erbB tumors. Months 36-41.

Writing/publishing experimental data. Months 42-48.